(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 427 074 A2**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 90120652.4

(22) Date of filing: 27.10.90

(51) Int. Cl.5: C12Q 1/68, C12P 19/34, //C07H21/04,C12Q1/70, G01N33/53

(30) Priority: 09.11.89 US 433947
23.08.90 US 569992

(43) Date of publication of application:
15.05.91 Bulletin 91/20

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: MOLECULAR DIAGNOSTICS, INC.
400 Morgan Lane
West Haven, CT 06516(US)

(72) Inventor: Dattagupta, Nanibhushan
470 Prospect Street
New Haven, CT 06511(US)

(74) Representative: Dänner, Klaus, Dr. et al
c/o Bayer AG Konzernverwaltung RP Patente Konzern
W-5090 Leverkusen 1 Bayerwerk(DE)

(54) Nucleic acid amplification employing transcribable hairpin probe.

(57) Specific nucleic acid sequences are amplified through the use of transcribable hairpin probes. The probe comprises a single stranded self-complementary sequence which, under hybridizing conditions, forms a hairpin structure having a functional promoter region, and further comprises a transcribable sequence extending from the 5′ end of the hairpin sequence and a probe sequence which may be comprised in the transcribable 5′ sequence or in a sequence extending from the 3′ end of the hairpin sequence. The hairpin form of the probe is transcribable in the presence of a suitable RNA polymerase and appropriate ribonucleoside triphosphates (rNTPs). Amplification is accomplished by hybridizing the desired target nucleic acid sequence with the transcribable probe, transcribing substantially only probe which has become hybridized to the sequence of interest, and allowing transcription to proceed until a desired amount of RNA transcription product has accumulated. Optional second stage amplification of the RNA transcripts can produce higher levels of amplification. The amplification method is particularly useful in assays for the detection of particular nucleic acid sequences.

EP 0 427 074 A2

looped structures whose shape resembles a common hairpin. Such hairpin structures are known to occur naturally in many organisms, particularly in RNA secondary structures, however, their functional role is at this point not well established. The physical chemistry of hairpin structures has been described - Cantor and Schimmel, Biophysical Chemistry, Part III, p. 1183, M.R. Freeman & Co. (San Francisco 1980).

The literature on this subject is incomplete and contradictory. For example, there are predictions that hairpins may provide a transcription termination signal - Jendrossek et al, J. Bacteriol. 170:5248 (1988) and Walker et al, Biochem. J. 224:799 (1984). Hairpin structures resembling known rho dependent transcription termination signals have been observed following the unc operon and glms of E. coli. On the other hand, pallindromic sequences capable of forming stable hairpin forms have been found around the transcription initiation site of beta amyloid precursor gene - La Fauci et al, Biochem. Biophys. Res. Commun. 159:297 (1989).

The use of hairpin structures in the synthesis of DNA from oligonucleotides and in the labeling of oligonucleotides is proposed in European Patent Publication 292,802 and by Sriprakash and Hartas, Gene Anal. Techn. 6:29-32 (1989). In addition, Krupp and Söll, in FEBS Letters 212:271 (1987) and in "Nucleic Acid Probes", ed. Symons (CRC Press, Bacon Raton, FL, 1989) pp. 21 & 22, describe the use of a hairpin structure to make labeled RNA transcripts from an M13 vector/T7 RNA polymerase system.

SUMMARY OF THE INVENTION

The present invention provides a method and means for amplifying a particular nucleic acid sequence of interest (target sequence) using a probe which is inherently transcribable, and accordingly, capable of providing multiple copies of RNA corresponding in base sequence to (i) a portion or the entirety of the target sequence or iii) a preselected sequence distinct from the target sequence. The transcribable probe has three principal parts, (1) a single stranded self-complementary sequence capable of forming, under suitable hybridizing conditions, a hairpin structure having a functional promoter region, and (2) a transcribable sequence extending from, and forming part of the same nucleic acid molecule with, the 5′ end of the self-complementary sequence, and (3) a single stranded probe sequence substantially complementary to the sequence of interest. The probe sequence can be comprised in the 5′ transcribable sequence or can be part of a sequence extending from the 3′ end of the hairpin sequence, in which latter case, it is not substantially complementary to the 5′ transcribable sequence.

Under suitable hybridizing conditions, the self-complementary region of the probe forms a looped, self-hybridized structure commonly referred to as a hairpin loop, or simply, hairpin. The base sequence of the self-complementary region is selected such that upon formation of the hairpin, a desired double stranded promoter sequence is formed operably linked to the transcribable region. Thus, the hairpin form of the probe as such is transcribable in the presence of a suitable RNA polymerase and the required ribonucleoside triphosphates (rNTPs). Where the probe sequence is comprised in the 5′ transcribable sequence, the sequence of bases in the RNA transcription product will accordingly be complementary with the probe sequence. Since, in such case, the probe sequence is complementary with the target sequence, transcription of the hairpin probe produces multiple RNA transcripts having the same base sequence as the target nucleic acid. Where the probe sequence is comprised in a sequence extending from the 3′ end of the hairpin region, the transcribable sequence can be selected to provide an RNA transcript having any useful sequence, for example, a sequence that is common for a series of probes for detecting a series of target sequences in order to enable use of a common, or universal, detection probe and system.

After hybridizing the target sequence with the present probe in a liquid mixture, the next step in the method is the transcription of probe which has become hybridized with the target without substantial transcription of any probe which is not hybridized to the select target sequence (unhybridized probe). Since the probe is transcribable irrespective of whether it is hybridized or unhybridized, it is necessary, in order to amplify and/or detect the target sequence specifically, to distinguish hybridized probe from unhybridized probe. Any suitable scheme may be applied for this purpose.

For example, one approach is based on separation of hybridized from unhybridized probe and then adding the polymerase and rNTPs only to the separated hybridized probe fraction. Another approach involves the use of a second probe which is complementary to a second sequence in the nucleic acid to be amplified. The base sequence of the second probe is selected such that its 3′ end is within ligating distance of the 5′ end of the transcribable probe when both such probes are hybridized with the target nucleic acid. After hybridization, the 3′ end of hybridized second probe is suitably ligated to the 5′ end of hybridized transcribable probe. Thus, transcription of the combined sequence encompassed by the now joined

lines BP representing the base pair bonds formed between the self-complementary sequences. Sequences a and $\bar{a}$ are selected in order that the resulting looped region A thereby comprises a functional promoter or transcription initiation site. The second principal part of the probe of the present invention is transcribable sequence c which is operatively linked directly or through an intervening sequence b to the 5′ end of promoter region A. The third principal part of the probe is a probe sequence that is selected to be hybridizable with $\bar{a}$ target sequence to be amplified or detected and can be comprised in transcribable sequence c or as part of sequence e extending from the 3′ end of the hairpin region. The probe can, in general, additionally comprise 5′ and/or 3′ flanking sequences such as are illustrated in Figs. 1 and 2 as sequences d and e.

The hairpin form of the probe as depicted in Fig. 2 is transcribable in the presence of a cognate polymerase and the rNTPs required for 3′-5′ transcription of transcribable sequence c and any intervening sequence b. Depending upon the concentration of probe present and reaction conditions, a functionally transcribable dimeric form can also be formed (Fig. 3). Self-complementary region A can potentially hybridize not only with itself, but also with a second probe molecule to form a dimer through hybridization of complementary regions a and a' on separate probe strands. Such dimer, like the hairpin form depicted in Fig. 2, comprises a functional promoter in region A and, in the presence of cognate polymerase and rNTPs, will produce 3′-5′ transcription of transcribable sequence c and any intervening and/or flanking sequences b and d. Accordingly, when reference is made herein to the "hairpin form" of the present probe, it will be understood that the functionally equivalent dimeric form is intended as well.

The promoter formed by hybridization of hairpin region A of the probe can be any double stranded nucleic acid (e.g., DNA or RNA) sequence that corresponds with, and is recognized by, a polymerase enzyme which, as is known in the art, binds to the promoter and thereby initiates 3′-5′ transcription. The length of the self-complementary sequences a and a' which form the promoter, while not critical to the operation of the present invention, will generally fall between about 7 and about 200 bases, and more commonly between about 10 and about 50 bases. In general, any promoter for which a suitable polymerase is known and available can be used in the present invention. Usually, the probe will be composed of DNA which is transcribable with DNA-dependent RNA polymerases, that is, polymerases that act upon a DNA template to produce RNA transcripts. However, RNA probes transcribable with RNA-dependent RNA polymerases (such as produced in certain viruses, e.g., retrovirus and picornovirus) can be used. Useful promoters include, among others, those recognized by RNA polymerases produced in bacteriophages, such as T7, T3, and SP6 phage.

Exemplary base sequences having promoter function with respect to such phage polymerases are (n = 2 to 50; the sequences are shown in their self-hybridized, hairpin form and do not necessarily represent the minimum sequences required for promoter function):

For T7

```
    TAATACGACTCACTATAG-3'
Tn
    ATTATGCTGAGTGATATC-5'
```

For T3

```
    ATTAACCCTCACTAAAGGGA-3'
Tn
    TAATTGGGAGTTAGCTTTCC-5'
```

For SP6

```
    CATACGATTTAGGTGACACTATAG-3'
Tn
    GTATGCTAAATCCACTGTCATATC-5'
```

The probe sequence, whether comprised in sequence c or e, can be any sequence which is capable of hybridizing with the target sequence of interest. As is known in the art, the degree of homology between the probe and target sequences will depend upon the specific needs of the user. Where a high degree of

be necessary or desirable to treat the test sample to release and/or extract the target nucleic acid for hybridization, and/or, when the target nucleic acid is presented in double stranded form, to denature and render such in hybridizable single stranded form by means well known in the art. Although not generally necessary, it can be desirable, such as to increase the overall efficiency of hybridization, to subject the target nucleic acid to restriction digest.

Since the present probe is designed in such a manner that it is capable of undergoing transcription whenever it is in the hairpin form, whether or not hybridized to the target, it is necessary to take steps to limit transcription or detection substantially only to probe that hybridizes with the target sequence. Any number of formats can be devised for the purpose of transcribing probe that has become hybridized with the target without transcribing substantially any of the hybridized probe. Approaches that are particularly advantageous are methods based on separation of hybridized probe from unhybridized probe, methods based on the use of a second probe which is ligatable to the transcribed 5′ end of the probe only if hybridized with the target, and methods based on use of a restriction enzyme which digests only hybridized probe to release a transcribable product.

Formats which are based on the separation of hybridized from unhybridized probe are generally illustrated by the Fig. 4 diagram. Hybridization of the present hairpin probe with the target sequence present in a sample of nucleic acids produces hybrids (I) in which a probe sequence c in the probe is hybridized with its complementary target sequence $\bar{c}'$. In Fig. 4, the target nucleic acid is shown with flanking regions f′ and g′ in addition to the target sequence $\bar{c}'$ and, the transcribable probe is depicted without regions flanking the hairpin region A or the probe sequence c, although, as discussed herein, other variations are also possible. Further, although not shown, these formats are also applicable where the probe sequence is comprised in a sequence extending from the 3′ end of the hairpin loop. After separation of unhybridized probe and addition of the polymerase and rNTPs, transcription proceeds with the generation of multiple transcripts having the combined sequence $\bar{b}'\bar{c}'$ (where b′ is complementary to b).

Numerous formats are possible for separating hybridized from unhybridized probe. Many such techniques are well known in the literature and others will be devised in the future. Any such technique can be applied to the present invention. After separation, the hybridized probe fraction will be contacted with the appropriate polymerase and the rNTPs required for transcription. Following are descriptions of just a few of the possible separation schemes that can be used in the present invention for this purpose.

(1) Immobilization of target - In this approach, the target sequence is immobilized either before or after hybridization with the transcribable probe and resulting immobilized hybrids are readily separated from unhybridized probe. As is well known in the art, target nucleic acid can be immobilized prior to hybridization by such techniques as noncovalent or covalent attachment to a suitable solid phase, e.g., nitrocellulose or activated nylon membranes or reactive or adsorbent latex particles. Immobilization can also be accomplished by hybridization with a separation probe, that is, a probe which is immobilized or is immobilizable and comprises a sequence hybridizable with a sequence in the target nucleic acid which is nonoverlapping with the sequence that is hybridizable with the transcribable probe. In use, dual hybridization occurs with the target nucleic acid becoming hybridized to both the separation probe and the transcribable probe. The resulting immobilized hybrids are readily separable from unhybridized transcribable probe.

(2) Modifying the target for post-hybridization immobilization - Rather than immobilizing the target sequence directly, it is known to modify the target prior to hybridization in such a manner that the target can subsequently serve as a site for immobilization. This also permits hybridization to take place under favorable solution kinetics. The target can be modified to be immobilizable in a number of known ways. Normally, a reactive site is introduced into the target which is capable of forming a stable covalent or noncovalent bond with a reaction partner. Immobilization can then be accomplished at the desired time by adding an immobilized form of the reaction partner to bind with the modified target strand. Typical reaction partners are ligands such as biotin and haptens which are capable of specific binding with avidin and anti-hapten antibodies, respectively.

Modification of the target stand in a sample can be accomplished in several different ways as well. One particularly useful approach involves the introduction of a suitable reactive site into the target strand by reaction with a reactive site-functionalized nucleic acid binding agent such as photoreactive intercalating agents (for example, see U.S. Pat. No. 4,737,454). Particular examples of such modifying agents are bifunctional conjugates comprising a photochemically reactive form of a psoralen coupled to a ligand such as biotin or a hapten.

(3) Use of anti-hybrid antibody reagents -Another known method for separating hybrids from unhybridized probe involves the use of antibody reagents which are prepared or selected to be selective for binding hybrids over single stranded nucleic acids. A variety of such anti-hybrid reagents are known in the literature, including anti-DNA/RNA and anti-RNA/RNA antibodies (U.S. Pat. No. 4,833,084 and European

quence. Thus, resulting transcript products will be uniquely identifiable to the hybridization event. A further modification involves the inclusion of a 3′ sequence that is complementary with a non-probe 5′ sequence on the hairpin probe or with a separate oligonucleotide such that, upon hybridization, multimers of the hairpin probe are formed by linkage of individual hairpin probes by the complementary sequences in their 3′, 5′, and/or the oligo sequence. Release of such multimer transcribable product occurs only when hybridization with the target sequence creates the required restriction site in the hybridization products.

Transcription is initiated by addition of polymerase and the required rNTPs to the liquid mixture that contains the hybrids comprising the transcribable probe. Under suitable conditions, the synthesis of RNA transcripts will proceed in a continuous manner providing that sufficient amounts of rNTPs are present. Normally, a ribonuclease inhibitor will be included in the transcription reaction mixture in order to avoid undesirable degradation of RNA transcripts by any ribonuclease contamination. Transcription is allowed to proceed for a predetermined period of time or until a detectable or desirable amount of RNA transcript has accumulated. The amount of RNA transcript produced in a given period of time will be proportional to the amount of target sequence present in the original sample. The accumulated transcription product thus serves as an amplification of the target sequence. Transcription can then be terminated by any conventional means such as inactivation of the polymerase or removal of reactants from the mixture.

Further amplification of the RNA transcription products can be accomplished in a number of ways, for example, by the use of replicases such as Qβ replicase or replicase from brome mosaic virus. Also, a separate set of hairpin probe/second probe pairs can be used where the hairpin probe (distinct from the original hairpin probe) and the second probe hybridize with adjacent sequences in the RNA transcription product. After hybridizing the hairpin probe/second probe pairs to transcripts, the hybridized pair is ligated to form transcribable nucleic acids which themselves will produce additional RNA transcript in the presence of the polymerase. Further, the RNA transcripts can be produced to contain a site for immobilization (e.g., by use of ligand, e.g., biotin or hapten, modified rNTPs and immobilization of resulting transcripts by addition of an immobilized form of a ligand binding partner, e.g., avidin or an anti-hapten antibody, respectively), and after being separated from the mixture can be hybridized to a further probe to introduce a promoter site for further transcription. After a few cycles, more than a million-fold amplification is possible.

The following methods in particular are useful for providing a second stage amplification:

(1) Displacing probe from hybrid by RNA - The promoter probe is hybridized with its complementary DNA which is immobilized onto a solid support or hybridized to an inmobilizable support. The immobilized or immobilizable support is brought into contact with the product RNA under conditions of specific hybridization. This releases the transcribable probe since RNA will hybridize instead of the DNA because of the stability difference between the RNA-DNA hybrid and DNA-DNA hybrid. After the first stage of transcription, the RNA polymerase activity is destroyed by heating before the mixture is reacted with the immobilized DNA hybrid support under conditions of strand displacement. For every molecule of RNA, one molecule of promoter probe will be produced under the most ideal conditions. By cycling the system it is possible to use RNA to produce more and more transcribable probes and hence secondary amplification of the system. For more effective strand displacement, branch migration can be utilized. In this manner, the displacing DNA molecule has on its 3′ or 5′ end an unhybridized single stranded region where a corresponding part of the RNA initiates RNA-DNA hybrid formation.

It has been assumed that a single strand branch migration phenomenon is involved in transcription of a double stranded DNA. A newly synthesized strand of RNA replaces a DNA strand of the same sequence. The migration rate of a single stranded branch is estimated to be faster than 1,000 base pairs per second. A double stranded branch migration has also been described. This process is about 6,000 base pairs per second at 37° C (Biophysical Chemistry, Cantor & Schimmel, Freeman publication, San Francisco, 1980, vol. III, pp. 1238-1239). From this information it can be easily estimated that a 20 base branch migration displacement should be a very rapid process without any high temperature treatment.

(2) Displacing one strand of the probe - This method is similar to the previous method except that the released probe is not the complete probe, but rather one strand of the probe with one strand of the promoter. Only addition of the other strand makes it transcribable.

(3) Displacing ligatable linker - This method is also similar to the previous methods, however, the released fragment acts as a linker for the ligation of two portions of the promoter probes.

(4) RNA mediated ligation - This method involves the use of RNA product to form a bridge over which ligation of two DNA fragments can occur and is characterized by the advantage of being capable of being performed in solution.

(5) Capture recycling - This method uses ligand-modified (e.g. biotinylated) RNA product of the first transcription as the capturing agent for the promoter probes. At the time of the initial transcription, biotinylated UTP is mixed with the other nucleoside triphosphates for the initial transcription. The biotinylat-

C.P.H. Vary (1987) Nucleic Acids Res. 15:6883-6897).

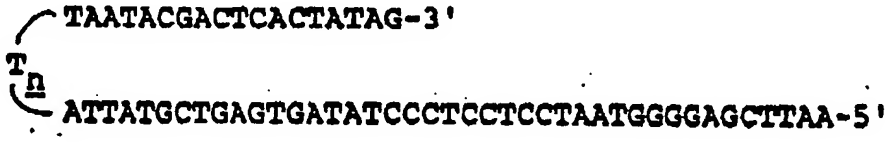
Of course, since the transcription system can be designed to produce transcripts of a specified size, in such case they can be readily identified by size resolution methods such as gel electrophoresis.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

EXAMPLE 1

Demonstration of transcription of hairpin probe

Oligonucleotides having the sequence shown below were synthesized (Applied Biosystems, Foster City, CA, USA, Model 380B oligonucleotide synthesizer) for n = 5 and 7 using reagent supplied by the manufacturer for a phosphoramidite-type synthesis:



The oligonucleotides were purified on 20% denaturing polyacrylamide gels by electrophoresis. The purified materials were tested for their ability to produce RNA transcripts in the presence of T7 bacteriophage RNA polymerase under the conditions defined below.

Mixtures (in a 25 microliter final volume) containing 1 mM ATP, GTP, CTP, and UTP and 2 microcurie of ^{32}P-labeled CTP (3000 curie/mM) 70 units of T7 RNA polymerase (Pharmacia, Milwaukee, WI, USA), and 1 ng of the template oligonucleotides, in 40 mM Tris-HCl (pH 8.1 at 37°C), 1 mM spermidine, 5 nm dithiothreitol, 50 g/ml bovine serum albumin, 0.01% (v/v) Triton X-100, and 80 mg/ml polyethylene glycol (8000 MW) were incubated at 37°C for 3 hours. The product was then analyzed by polyacrylamide gel electrophoresis and autoradiography.

Fig. 7 shows an autoradiogram of a gel on which 10 picogram equivalents were loaded. Lanes 10, 50 and 100 indicate the amount of starting oligonucleotide in picograms used in the transcription. After transcription, all, one-fifth and one-tenth of the material was loaded for analysis. The results show production of similar amounts of RNA from similar amounts of template irrespective of initial concentration.

EXAMPLE 2

Use of a transcribable hairpin probe to detect a target nucleic acid sequence

A. Hybridization of immobilized sample nucleic acids

Transcribable hairpin oligonucleotides are prepared as in Example 1 to have the following different probe sequences added to their respective 5′ ends:
5′-AGA TTT TCT AGA CAT CTT-3′, and
5′-CAA GCA AGT TTA GCT CTC TCT-3′
The above probe sequences are specific to a membrane protein in the bacterium Chlamydia trachomatis. Nucleic acids from a sample to be tested for the presence of C. trachomatis are immobilized onto a solid support such as polystyrene latex. The immobilized sample nucleic acids are then hybridized with the transcribable probes under conventional conditions (Maniatis et al (1982) Molecular Cloning, Cold Spring Harbor). After washing to remove unhybridized probes, the hybridized probes are released into solution by

Such technique is applicable to viruses such as HIV and HRV, and also to bacteria through detection of ribosomal RNA. The following example relates to the detection of human rhinovirus (HRV 14) in a sample. A similar method can be used for the detection of other RNA analytes. The DNA probe sequence has a duplex promoter sequence and a complementary probe sequence as follows (the probe sequence is underlined and comes from Bruce et al (1989) Arch. Virol. 105:179-187):

5′-GAGGCCGGGGACTTACGATGCCCTATAGTGAGTCGTATTATTTTTTAATACGACTCACTATAG- 3′

Anti-DNA/PNA antibody is prepared according to the method of Boguslawski et al, J. Immunol. Meth. (1986) 89:123-130. The IgG fraction of the antibody is immobilized onto microspheres by incubating a mixture of antibody and a microspheres suspension at 4°C overnight according to the method of Colvin et al, Microspheres: Medical and Biological Applications, ed. Rembaum and Toekes, CRC Press (Boca Raton, FL 1988) pp. 1-13.

Suspensions containing viruses are treated with proteinase K and then extracted with phenol: chloroform:isoamyl alcohol (50:50:2) and the RNA precipitated with ethanol. The extracted RNA is then hybridized with the promoter-containing hairpin probe in a solution containing SSPE (10 mM sodium phosphate buffer, pH 7.4, 0.15 M sodium chloride, 1 mM EDTA), 5% polyethylene glycol, 0.2 mg/ml salmon sperm DNA at 40°C for 30 minutes. The antibody-immobilized microspheres are also treated with the same solution to saturate any DNA binding sites. After hybridization, the mixture is combined with the microsphere suspension and washed with the hybridization buffer and then with the transcription buffer at 37°C to remove any unhybridized probe. The microspheres with captured hybrids are then transcribed as in Example 1 and the products are analyzed by gel electrophoresis. Alternatively, the microspheres carrying hybrids can be heated to release the probes which can be transcribed and analyzed for the detection of analyte. The amount of product RNA is correlated with the amount of analyte RNA.

EXAMPLE 4

Amplification involving second probe and ligation in place of separation

The probe regions of the two probes used in this method (previously described in detail with reference to Fig. S) are designed such that they will hybridize with the target sequence (in this example, a chlamydial plasmid sequence) in mutually exclusive regions but with the phosphorylated 5′-end of the promoter-containing hairpin probe (see sequence below) being juxtaposed with the 3′-end of the second probe (see sequence below):

Sequence of hairpin probe (SJH P101A):

5′p-TCTCTCTCGTAATCCTCCCTATAGTGAGTCGTATTATTTTTTAATACGACTCACTATAG- 3′

Sequence of second probe (PCL5):

5′-GTCTACCACCAAGAGTTGCAAA-3′

The probes are prepared in an automated DNA synthesizer. The SJH P101 probe is kinased with polynucleotide kinase and ATP according to the method of Maniatis et al, supra, p. 122.

A sample of whole genomic chlamydial DNA containing the cryptic plasmid (Black et al (1989) Current Microbiology 19:67-74) is digested with SaCl restriction enzyme and the digest is heated to 100°C for 5 minutes for denaturation. After heating, the sample is chilled on ice. The denatured, digested DNA is then hybridized for 10 minutes with the SJH P101A and PCL5 probes at 45°C in transcription buffer as in example 1. After hybridization, 20 units of T4 ligase (Boehringer Mannheim, Indianapolis, IN, USA) and ATP (final concentration of 10 mM) are added and the ligation carried out at 37°C for 60 minutes. After ligation, the nucleic acids are optionally purified by phenol extraction and precipitated with ethanol. Transcription then proceeds in transcription buffer with 70 units of T7 RNA polymerase and the analysis as performed as in Example 1.

EXAMPLE 5

Further amplification of the amplified transcription products - second stage amplification

and (3) a single stranded probe sequence substantially complementary to said sequence of interest,

(b) transcribing said probe which has become hybridized with said sequence of interest without transcribing substantially any of the unhybridized probe, such transcription taking place under hairpin-forming hybridizing conditions, and

(c) allowing the resulting transcription of the probe sequence in said hybridized probe to take place for a predetermined period of time to accumulate the resulting RNA transcription product as an amplification of said sequence of interest.

2. The method of claim 1 wherein said probe sequence is comprised in said transcribable 5′ sequence.

3. The method of claim 1 wherein said probe sequence is comprised in a sequence extending from the 3′ end of said self-complementary sequence and which is not substantially complementary to said 5′ transcribable sequence.

4. The method of any of claims 1 to 3 wherein the resulting RNA transcription product is further amplified in a second stage amplification.

5. The method of any of claims 1 to 4 wherein said polymerase is a DNA-dependent RNA polymerase, preferably T7, T3, or SP6 bacteriophage RNA polymerase, and preferably wherein said promoter region comprises the following base sequence shown in its hairpin form:

for T7,

```
 TAATACGACTCACTATAG-3'
Tn
 ATTATGCTGAGTGATATC-5';
```

for T3,

```
 ATTAACCCTCACTAAAGGGA-3'
Tn
 TAATTGGGAGTTAGCTTTCC-5'; or
```

for SP6,

```
 CATACGATTTAGGTGACACTATAG-3'
Tn
 GTATGCTAAATCCACTGTCATATC-5';
```

wherein n is an integer from 2 to 50 inclusive.

6. A method for detecting a particular nucleic acid sequence in a test sample, comprising the steps of:

(a) hybridizing said sequence of interest in a liquid mixutre with a transcribable probe comprising (1) a single stranded self-complementary sequence capable of forming, under hybridizing conditions, a hairpin structure having a functional promoter region, (2) a transcribable sequence extending from the 5′ end of said self-complementary sequence, which 5′ sequence is transcribable in the presence of an RNA polymerase and required ribonucleoside triphosphates her said hairpin-forming hybridizing conditions, and (3) a single stranded probe sequence substantially complementary to said sequence of interest,

(b) transcribing said probe which has become hybridized with said sequence of interest without transcribing substantially any of the unhybridized probe, such transcription taking place under haiprin-forming hybridizing conditions, and

(c) allowing the resulting RNA transcription of the probe sequence in said hybridized probe to take place for a predetermined period of time to accumulate the resulting transcription product as an amplification of said sequence of interest and

(d) detecting the accumulated RNA transcription product.

7. The method of claim 6 wherein said probe sequence is comprised in said transcribable 5′ sequence.

8. The method of claim 6 wherein said probe sequence is comprised in a sequence extending from the 3′ end of said self-complementary sequence and which is not substantially complementary to said 5′ transcribable sequence.

9. The method of any of claims 6 to 8 wherein the resulting RNA transcription product is further amplified in a second stage amplification.

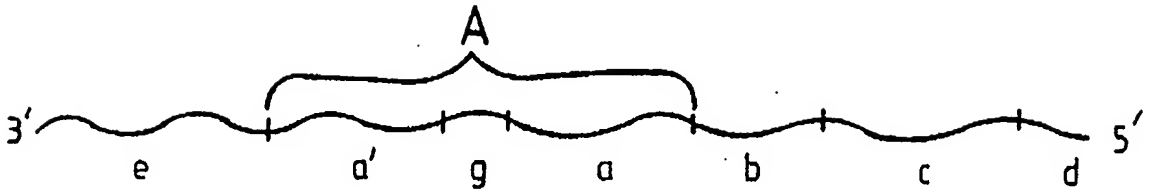


FIG.1

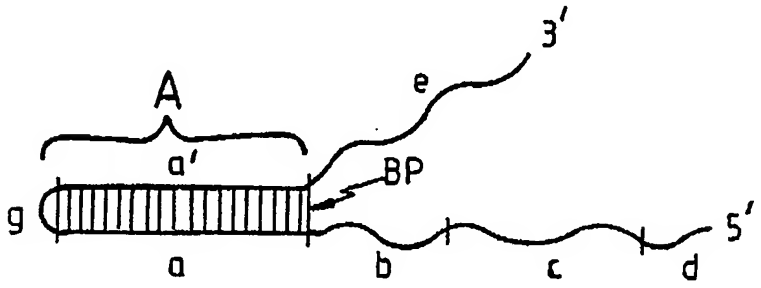


FIG.2

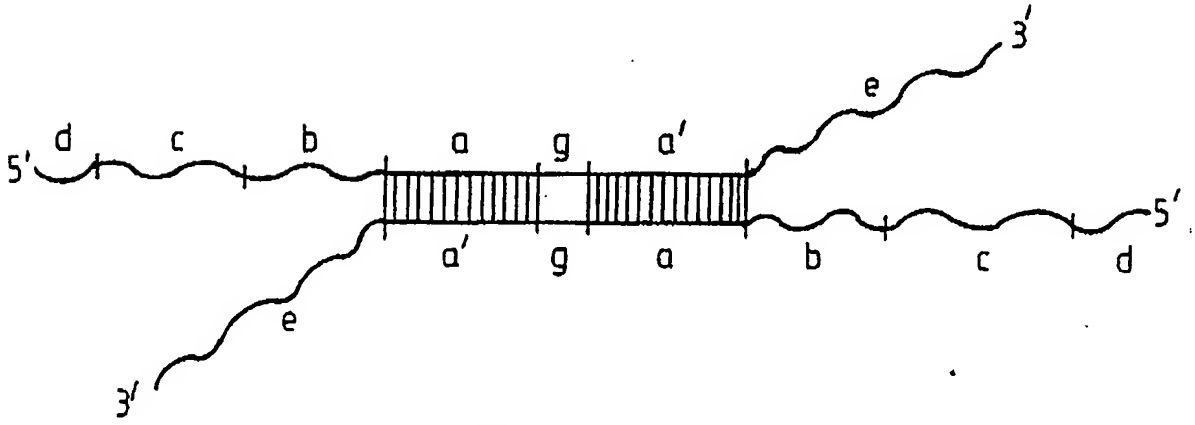


FIG.3

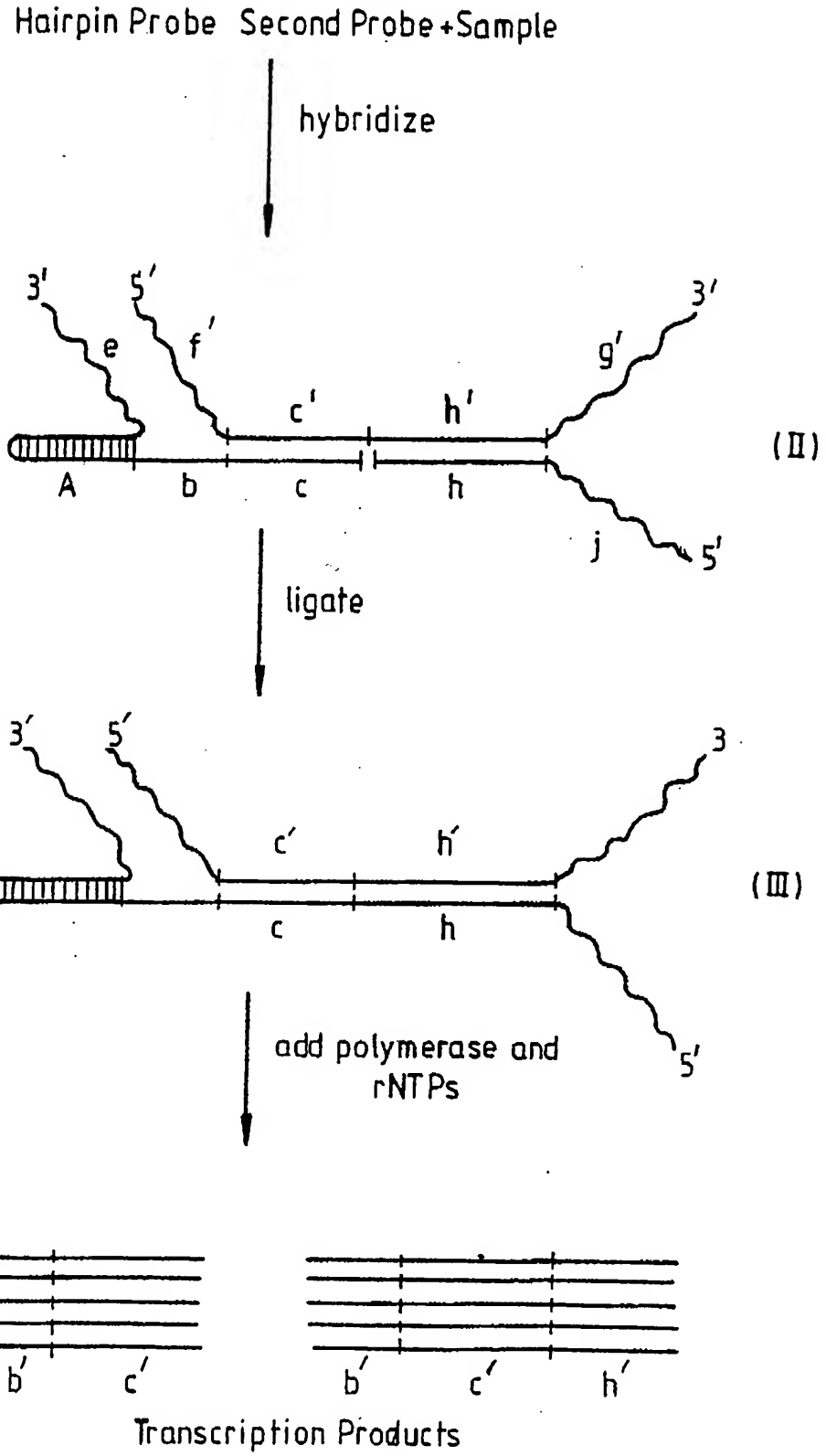

Hairpin Probe
Second Probe+Sample
hybridize
3'
5'
e
f'
c'
h'
g'
3'
A
b
c
h
j
5'
(II)
ligate
3'
5'
3
c'
h'
c
h
(III)
add polymerase and
rNTPs
5'
b'
c'
b'
c'
h'
Transcription Products

FIG.5

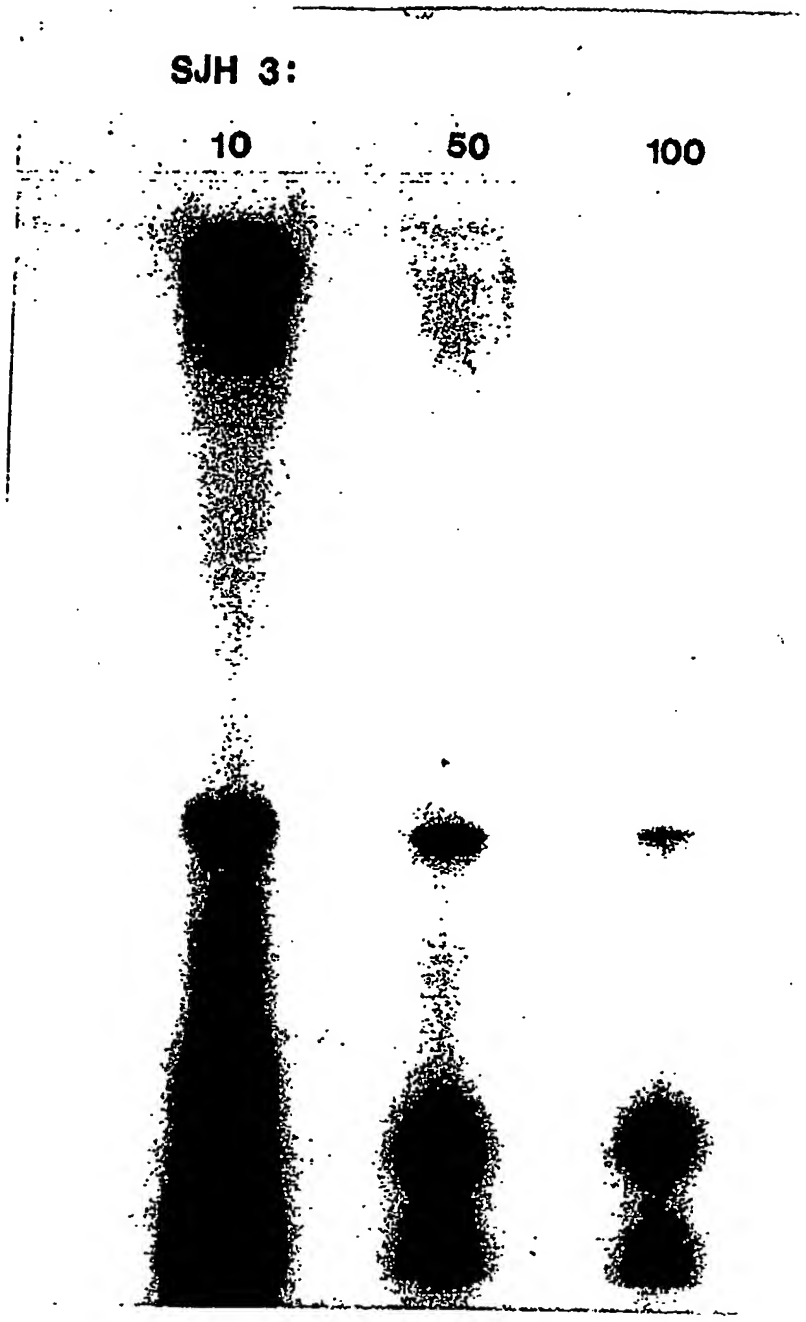


FIG. 7

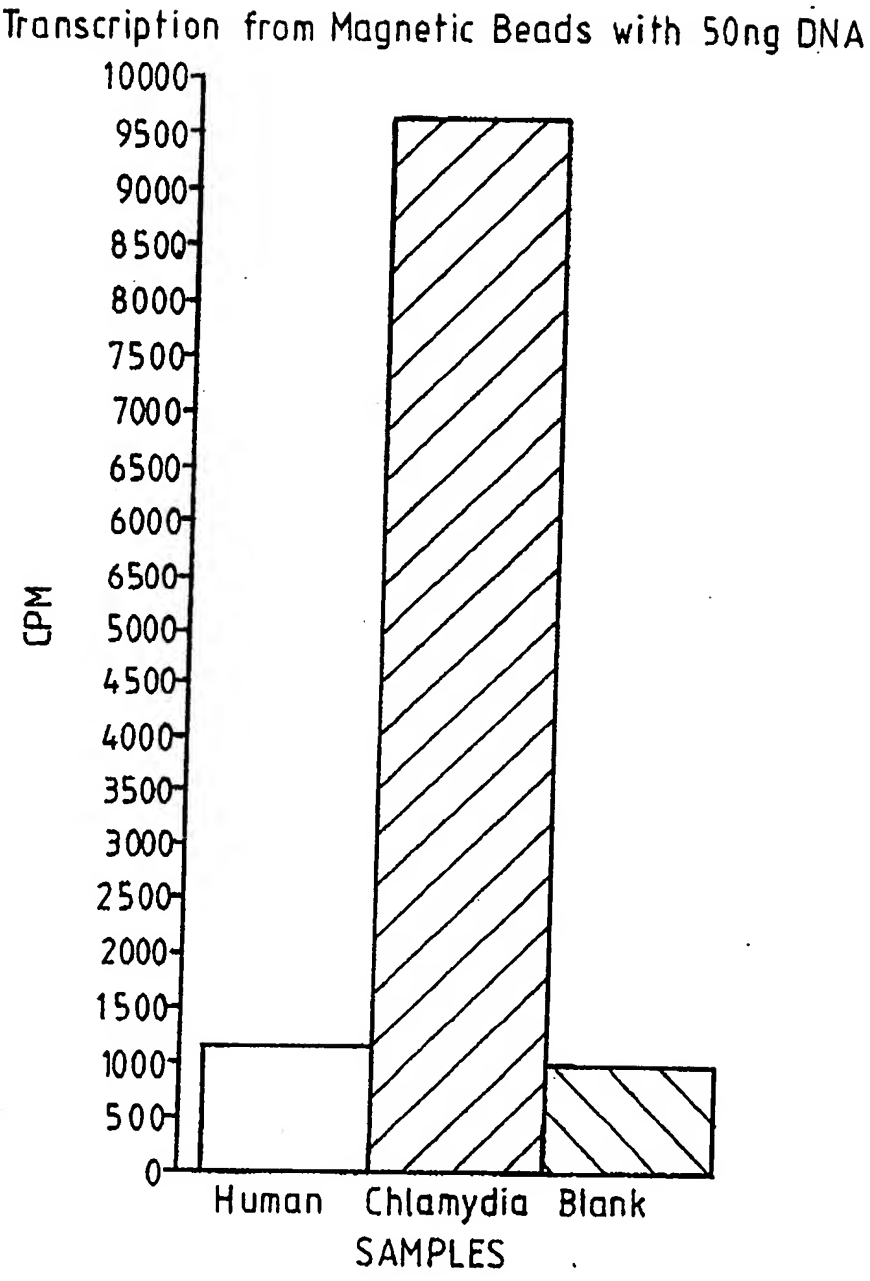

Transcription from Magnetic Beads with 50ng DNA
CPM
10000
9500
9000
8500
8000
7500
7000
6500
6000
6500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Human
Chlamydia
Blank
SAMPLES

FIG. 9

(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

(11) Publication number: 04304900 A
(43) Date of publication of application: 28.10.1992

(51) Int. Cl C12Q 1/68

(21) Application number: 03093260
(22) Date of filing: 29.03.1991

(71) Applicant: TOYOBO CO LTD
(72) Inventor: AONO TOSHIYA
TAKARADA YUTAKA
SHIBATA HIDEJI

(54) DETECTION OF TARGET NUCLEIC ACID SEQUENCE AND REAGENT KIT THEREFOR

(57) Abstract:

PURPOSE: To detect the target nucleic acid while suppressing non-specific reaction by preparing a probe cyclizing in the presence of the target nucleic acid, hybridizing the probe to the target nucleic acid in a specimen, using the cyclized material as a template and determining the amount of the single-stranded nucleic acid having a primer sequence complementary to the template.

CONSTITUTION: A straight-chain probe nucleotide B having a sequence designed to cause the cyclization as a result of the presence of the target nucleic acid sequence A in a specimen is hybridized to the target nucleic acid sequence A by using a primer nucleotide C having a sequence at least partly complementary to the straight-chain probe nucleotide B. The straight-chain probe nucleotide B is cyclized by this process. The produced cyclic probe nucleotide B' is used as a template and a single-stranded nucleic acid having a sequence consisting of repetition of sequences complementary to the template is produced by utilizing the primer nucleotide C. The target nucleic acid sequence A in the specimen can be detected by the produced single-stranded nucleic acid.